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Table of Contents

Front Cover	1
Standard Form 298	2
Table of Contents	3
Introduction	4
Body	5
Key Research Accomplishments	8
Reportable Outcomes	8
Conclusions	8
References	9
Appendices	NA

Proposal Title: Breast Cancer Biomarkers Based on Ductal Lavage Specimens

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Introduction

The recent progress in defining the early premalignant phases of breast cancer has made primary prevention a real possibility. Women with a familial risk of breast cancer are a particularly good population to target for chemopreventive approaches to reduce breast cancer incidence because of their significantly increased risk and their significantly earlier age of onset of the disease. The increased risk for both initial and contralateral breast cancer associated with a family history is two- to four-fold (1). Women who are carriers of a mutation in the *BRCA1* or *BRCA2* genes may have a lifetime risk of breast cancer as high as 85% (2-4). Furthermore, women who are *BRCA1* mutation carriers are more likely to develop tumors that are estrogen and progesterone receptor negative and are less likely to benefit from Tamoxifen as a chemopreventive agent (5,6).

Preliminary studies performed in our laboratory have capitalized on the protective effect of pregnancy reported both in women (7-9) and in experimental studies (10-13). Human chorionic gonadotropin (hCG), a glycoprotein hormone secreted early in pregnancy by the developing embryo, has been suggested as one source of protection associated with pregnancy (14-16). Suppression of mammary carcinogenesis similar to, or even more efficient than that induced by pregnancy has been achieved by treatment of young virgin rats with hCG, given as daily intraperitoneal injections for 21 days, a time that in the rat model is equivalent to the length of a full term pregnancy (14-17). Other studies, funded through non-DOD sources (see below) have further demonstrated that hCG treatment of rats previously exposed to DMBA inhibits the progression of preneoplastic lesions, such as intraductal proliferations and carcinomas in situ (17-20). This indication of a direct effect of hCG on the mammary epithelium has been further confirmed by *in vitro* hCG treatment of MCF-10 and MCF-7 cells, normal and neoplastic breast epithelial cell lines respectively (21). In these cells, hCG inhibits growth, depresses cell proliferation, lengthens the G1 phase of the cell cycle, and induces the synthesis of α and β inhibin, a non-steroidal glycoprotein belonging to the TGB- β family with demonstrated tumor suppressor activity (22-25). These observations indicate that hCG acts both through ovarian stimulation and directly on individual mammary cells to inhibit cancer cell growth by activating an autocrine/paracrine loop mediated by inhibin. Based on the strength of this preclinical data, a pilot clinical trial was conducted to determine whether hCG administered to breast cancer patients would reduce tumor burden through the mechanisms described above. Twenty-five postmenopausal women with palpable primary breast cancers >1.5 cm were eligible. After confirmation of the diagnosis by core biopsy, patients were randomized to receive 500 μ g of recombinant human chorionic gonadotropin (r-hCG) by intramuscular injection every other day times seven, or saline placebo. At the completion of the study treatment, all patients underwent mastectomy. Pre- and post-treatment tumor specimens were examined for rate of cell proliferation, estrogen and progesterone receptor expression, and immunoreactivity for inhibin. Treatment with r-hCG induced a statistically significant decrease in rates of cell proliferation and down regulation of ER and PR expression in tumor cells. At the same time, a significant increase in immunoreactivity for inhibin was observed in the neoplastic cells. No systemic changes in ovarian steroid or pituitary hormones were observed, suggesting that the effects of hCG are

organ specific (26). Our experimental *in vitro* and *in vivo* systems have demonstrated that hCG produces the following effects:

- 1) prevention of the initiation of chemically-induced mammary carcinomas;
- 2) inhibition of the progression of mammary carcinomas;
- 3) induction of differentiation of the mammary gland, inhibition of cell proliferation and increased apoptosis; and
- 4) induction of the synthesis of inhibin.

Human chorionic gonadotropin (hCG) has been used clinically for many years for the treatment of male and female infertility, corpus luteum insufficiency, habitual or threatened abortion, hypogonadism and cryptorchidism in the male, and weight reduction (27-29). It is well tolerated without significant toxicities and therefore offers enormous promise as a chemopreventive agent for women with a familial/genetic risk for breast cancer. However, the inability to predict in this high risk population who will develop breast cancer has required the implementation of broad, population-based strategies utilizing preventive measures that have significant side effects and require protracted treatment. Therefore, we are addressing the need of precisely identifying those women who should take a preventive agent, sparing others who will not develop the disease during their lifetimes. It is in this status of knowledge that we have developed a new paradigm for breast cancer prevention, the identification of the genomic and proteomic signatures that characterize the breast at high risk. The accomplishment of this goal has become feasible due to the recent development of ductal lavage (30-35), a noninvasive procedure that yields both cellular and non-cellular material for evaluation, high-throughput microarray (36,37) and proteomic (38-40) technologies, and laser capture microdissection (LCM) for obtaining selected populations of cells, and RNA amplification procedures, all available technologies whose combined use represent powerful tools for generating a vast amount of information on the genomic and protein profiles of the breast ductal system in a single experiment.

Body

A large body of literature has established the prognostic importance of atypical ductal epithelial cells found either in breast biopsy material or in nipple aspirates (30, 31). Ductal Lavage is a newer minimally invasive procedure developed to enhance the collection of ductal epithelial cells for cytological analysis. Ductal Lavage produces cellular material in 78% of patients, has a significantly higher yield of ductal cells than nipple aspiration alone, and detects the same rate of abnormal ductal cells as fine needle aspirate (FNA) with a much less invasive approach (34). This procedure is being performed by the PI in collaboration with Dr. M.B. Daly and A. Masny, R.N., M.P.H., nurse practitioner. A training protocol was been implemented for acquiring proficiency in the technique and for assessing the usefulness of cells and proteins obtained by ductal lavage (DL) and nipple aspirate fluid (NAF) respectively, for applying novel technologies for the genomic and proteomic analysis of the specimens. **A Protocol for the Training of Health Professionals in the Ductal Lavage Procedure and Scientists in the Analysis of Biomarkers in Ductal Fluid** was developed for the research project entitled: **Prevention of Breast Cancer in Young Women Known to be at High Risk Because of Previous Radiation Exposures.** This study was supported by the Tobacco Fund of the Pennsylvania Department of Health, FCCC Grant No. 69151-01 and FCCC IRB protocol # 02-814,

twenty volunteers were accrued between August 11,2003 and July 31, 2004. The objectives set for this study were to enroll 20 (twenty) healthy women to serve as volunteers for a Ductal Lavage training protocol; to determine the feasibility of obtaining nipple aspirate and ductal lavage, and to perform cytological, genomic, and proteomic studies in the nipple aspirate and ductal lavage obtained.

Conditions for subject eligibility (inclusion criteria) were developed following the guidelines of the Fox Chase Cancer Center Family Risk Assessment Program. Women were eligible if they met the following criteria: They were between the ages of 18 and 65; they had not been diagnosed with breast and/or ovarian cancer; they were at an increased risk for breast cancer based on family history, BRCA1/BRCA2 genetic test result, or a history of previous benign breast disease (atypical hyperplasia or Lobular Carcinoma in Situ (LCIS)); they were not pregnant or currently lactating; they were not taking Tamoxifen or Raloxifene or participating in the Study of Tamoxifen and Raloxifene (STAR) study; they were not on oral contraceptive, and they were not on hormone replacement therapy; they had not had a history of reduction mammoplasty or breast augmentation. Women of all racial and ethnic groups who met eligibility criteria were invited to participate.

Registration procedures and volunteer recruitment previously established were reinforced. Women who were considered eligible by review of their personal and family profile and their Gail score had been determined were contacted by phone to introduce the training protocol and to confirm eligibility. For those whose eligibility was confirmed, a clinic appointment was arranged. Informed consent was obtained for every volunteer prior to participating in the **Protocol for the Training of Health Professionals in the Ductal Lavage Procedure and Scientists in the Analysis of Biomarkers in Ductal Fluid**. A Data and Safety Monitoring Plan (DSMP) was developed and approved by the Fox Chase Cancer Center's Research Review Committee (RRC) and the Institutional Review Board (IRB). This plan includes reporting all adverse events immediately to the IRB by the Principal Investigator and review of safety and study conduct.

It was considered that the approval of the Protocol for Training Health Care Professionals in the Ductal Lavage Procedure and Scientists in the Analysis of Biomarkers in Ductal Fluid, " Submitted by Irma Russo, M.D., Fox Chase Cancer Center, Philadelphia, PA, Award Number DAMD17-98-1-8083, Surgeon General's Human Subjects Research Review Board (HSRRB) Log Number A-08047.2 grant would allow us to continue the collection of samples by ductal lavage (DL) and nipple aspirate fluid (NAF) and the genomic analysis of these samples. Until HSRRB approval was granted we decided to proceed to solve important methodological problems regarding the need to improve or set techniques for the isolation of pure epithelial cells using laser capture microdissection (LCM), to improve the procedures for RNA isolation and amplification, and to obtain reproducible cDNA microarrays. For these purposes we decided to utilize human breast epithelial cells maintained *in vitro* in our laboratory for reproducing in this system the same methodology planned to be applied when we obtain DL and NAF specimens for analysis. The report described below provides a detailed account of the methodology that was developed using the *in vitro* system for the purposes indicated above.

Laser Capture Microdissection (LCM) of Breast Epithelial Cells for RNA Extraction

The combined use of cytological smears with LCM (PixCell II System; Arcturus Engineering) allows the selection of specific epithelial cell populations without contamination with foam cells, macrophages, or inflammatory cells. For assessing the yield of RNA from fixed number of epithelial cells we performed a calibration assay by preparing cyto-smears with cultured normal breast epithelial cells for generating preliminary data before performing the procedure with actual ductal lavage specimens. The normal human breast epithelial cell line MCF-10F (41) was obtained from -80°C freezer, defrosted and counted on hemacytometer, viability of cells was recorded. Total of 3.7×10^6 cells with 90% viability were used.

1. After a wash with normal saline, the cells were fixed in nuclease free 50% ETOH for 20 min., then evenly distributed into 8 cyto-spin non adhesive slide, centrifuged at 1200 rpm at room temperature for 10 min. Each slide contained approximately 4.6×10^5 cells.
2. The cyto-spin preparations were air dried at room temperature for 2 min., then hydrated, stained with histostain, washed in double distilled water, dehydrated in increasing concentrations of ethanol, cleared in xylene for 5 min. and air dried for immediate use in LCM.
3. Cells were collected by LCM at the following concentrations: 20 cells, 200 cells, and 2000 cells. Each set consisted of two tubes.
4. RNA from each set of cells was extracted with PicoPure RNA Isolation Kit (Arcturus Engineering). Cells were mixed with 20 μl of RNA extraction buffer and incubated at 42°C for 30 min., then processed following the manufacturer's recommended procedures.
5. Purified RNA was DNAsed and measured by fluorometric quantitation with RibogreenTM, and a linear RT-PCR assay developed in our laboratory. In order to obtain enough RNA (20-40 μg) needed for microarray analysis, RNA was amplified using linear and PCR amplification (Table 1).

Table 1. Total RNA extracted from MCF-10F cells after LCM

<u>MCF10F Cells Number</u>	<u>Initial Total RNA (ng)</u>	<u>Total Volume (μl)¹</u>	<u>Amplified aRNA UV260/UV280</u>	<u>Concentration (ng/μl)</u>
<u>20</u>	<u>NA</u>	<u>10</u>	<u>1.35</u>	<u>NA</u>
<u>20</u>	<u>NA</u>	<u>10</u>	<u>1.33</u>	<u>NA</u>
<u>200</u>	<u>NA</u>	<u>10</u>	<u>1.35</u>	<u>NA</u>
<u>200</u>	<u>NA</u>	<u>10</u>	<u>1.37</u>	<u>NA</u>
<u>2000</u>	<u>21</u>	<u>10</u>	<u>1.94</u>	<u>2.1</u>
<u>2000</u>	<u>65</u>	<u>10</u>	<u>1.90</u>	<u>6.5</u>

¹PicoPure Extracted TRNA with 1 μl out of 10 μl total RNA

The housekeeping gene 18S was utilized for the RT-PCR analysis of Amplified RNA. A standard curve was generated, which expressed the following values (Table 2): Concentrations of 18S gene were compared with those of the breast epithelium specific genes fat milk globule membrane antigen and whey acidic protein gene.

Table 2. 18 S RT-PCR Measurement in Total RNA

<u>MCF10F</u> <u>Cells</u> <u>Number</u>	<u>Initial</u> <u>Total</u> <u>RNA (ng)</u>	<u>Total</u> <u>Volume (μl)¹</u>	<u>RT-PCR Volume</u> <u>(μl)</u>	<u>Concentration</u> <u>(ng/μl)</u>
<u>20</u>	<u>0.33</u>	<u>10</u>	<u>1</u>	<u>0.033</u>
<u>20</u>	<u>0.38</u>	<u>10</u>	<u>1</u>	<u>0.038</u>
<u>200</u>	<u>0.35</u>	<u>10</u>	<u>1</u>	<u>0.035</u>
<u>200</u>	<u>0.73</u>	<u>10</u>	<u>1</u>	<u>0.073</u>
<u>2000</u>	<u>19</u>	<u>10</u>	<u>1</u>	<u>1.9</u>
<u>2000</u>	<u>53</u>	<u>10</u>	<u>1</u>	<u>5.3</u>

¹with 1 μl out of 10 μl total RNA

Our results led us to conclude that RT-PCR is sensitive for detecting RNA from small numbers of LCM selected cells, being able to detect few copies of RNA, whereas the amounts of RNA obtained by linear amplification did not suffice for its use in microarray analysis.

Key Research Accomplishments

- Confirmation of the usefulness of the application of laser capture microdissection (LCM) to cytospin preparations for obtaining pure cell populations for RNA extraction
- Confirmation of the usefulness of RT-PCR RNA amplification for cDNA microarray analysis
- Confirmation of the usefulness of RT-PCR for gene expression level quantification

Reportable Outcomes

This study has allowed us to confirm the possibilities of using a minimal number of cells (20) and the utilization of RT-PCR instead of linear amplification for obtaining RNA in adequate amount and quality for performing cDNA microarray analysis. This step was significantly important to warrant that the breast epithelial cells from selected donors can be studied in the same fashion

Conclusions

The first year of this study has allowed us to establish the necessary baselines for utilizing the most useful technologies for RNA amplification, quantitation and gene analysis. Continuation of these studies will lead to fruitful results through genomic hierarchical cluster analysis and Bioinformatics for patient risk assessment when the adequate HSRRB approval by the DOD be obtained.

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